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Kyogo ITOH et al.
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For: NOVEL TUMOR ANTIGEN PROTEIN SART-3 AND TUMOR ANTIGEN PEPTIDES THEREOF

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10-242660	Japan	August 28, 1998

The undersigned declares further that all statements made herein of his own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment; or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issuing thereon.

Signed this 2nd day of July, 2007

Signature: 

Minori SAITO

Address: c/o AOYAMA & PARTNERS,
IMP Building, 1-3-7, Shiromi, Chuo-ku,
Osaka 540-0001 JAPAN

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Applicant: Kyogo ITOH

SUMITOMO PHARMACEUTICALS COMPANY, LIMITED

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Inventor:

Address:	2-25-9, Keyaki-dai, Kiyama-cho, Miyaki-gun, Saga-ken
Name:	Kyogo ITOH

Inventor:

Address:	277-1, Oaza Ohashi, Okawa-shi, Fukuoka-ken
Name:	Masanobu NAKAO

Applicant:

Identification No.:	596094371
Address:	2-25-9, Keyaki-dai, Kiyama-cho, Miyaki-gun, Saga-ken
Name:	Kyogo ITOH

Applicant:

Identification No.:	000183370
Name:	SUMITOMO PHARMACEUTICALS COMPANY, LIMITED
Representative:	Masaaki YOKOTSUKA

Agent:

Identification No.: 100107629

Patent Attorney:
Name: Toshio NAKAMURA

Phone No.: 06-466-5214

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Title of the invention: NOVEL TUMOR ANTIGEN PROTEIN SART-3 AND TUMOR ANTIGEN PEPTIDES THEREOF

What is claimed is:

1. A DNA encoding a protein having an amino acid sequence shown in SEQ ID NO: 1, or a protein variant having an amino acid sequence containing substitution, deletion, and/or addition of one or more amino acid residues of SEQ ID NO: 1, provided that the protein and the protein variant give rise to tumor antigen peptides that are capable of binding to an HLA antigen and being recognized by cytotoxic T lymphocytes.

2. A DNA consisting of a base sequence shown in SEQ ID NO: 2, or a DNA variant that hybridizes to the DNA under a stringent condition, provided that a protein produced and expressed by the DNA or the DNA variant gives rise to tumor antigen peptides that are capable of binding to an HLA antigen and being recognized by cytotoxic T lymphocytes.

3. An expression plasmid that contains the DNA of claim 1 or 2.

4. A transformant that is transformed with the expression plasmid of claim 3.

5. A process for producing a recombinant protein, which comprises culturing the transformant of claim 4, and recovering the expressed recombinant protein.

6. A tumor antigen protein that is produced by expressing the DNA of claim 1 or 2.

7. A pharmaceutical composition that comprises as an

active ingredient the DNA of claim 1 or 2, or the protein of claim 6.

8. A pharmaceutical composition for treating or preventing tumors, which comprises as an active ingredient the DNA of claim 1 or 2, or the protein of claim 6.

9. A tumor antigen peptide that is a partial peptide derived from the protein of claim 6, and that is capable of binding to an HLA antigen and being recognized by cytotoxic T lymphocytes, or a derivative thereof having the functionally equivalent properties.

10. The tumor antigen peptide of claim 9 wherein the HLA antigen is HLA-A24 or HLA-A2, or a derivative thereof having the functionally equivalent properties.

11. The tumor antigen peptide of claim 10, which is selected from those sequences, each comprising all or part of an amino acid sequence shown in any one of SEQ ID NOs: 3-24, or a derivative thereof having the functionally equivalent properties.

12. The tumor antigen peptide of claim 11, which comprises all or part of an amino acid sequence shown in any one of SEQ ID NOs: 3-9, or a derivative thereof having the functionally equivalent properties.

13. The tumor antigen peptide derivative of claim 12, which comprises all or part of an amino acid sequence wherein the amino acid residue at position 2 and/or the C-terminus in the amino acid sequence shown in any one of SEQ ID NOs: 3-9 is substituted by another amino acid residue.

14. The tumor antigen peptide derivative of claim 13, which comprises all or part of the amino acid sequence shown in any one of SEQ ID NOs: 25-31.

15. A pharmaceutical composition for treating or preventing tumors, which comprises as an active ingredient at least one substance selected from the tumor antigen peptides and derivatives thereof according to any one of claims 9 to 14.

16. An antibody that specifically binds to any one of the tumor antigen protein of claim 6, and the tumor antigen peptide or the derivative thereof according to any one of claims 9 to 17.

17. An antigen-presenting cell wherein a complex between an HLA antigen and the tumor antigen peptide or the derivative thereof according to any one of claims 9 to 14 is presented on the surface of a cell having antigen-presenting ability, which cell is isolated from a tumor patient.

18. A pharmaceutical composition for treating tumors, which comprises as an active ingredient the antigen-presenting cell of claim 17.

19. A cytotoxic T lymphocyte that specifically recognizes a complex between an HLA antigen and the tumor antigen peptide or derivative thereof according to any one of claims 9 to 14.

20. A pharmaceutical composition for treating tumors, which comprises as an active ingredient the cytotoxic T lymphocyte of claim 19.

Detailed explanation of the invention:

[0001]

Technical field to which the invention pertains:

The present invention relates to a novel tumor antigen protein, and tumor antigen peptides thereof. More particularly, it relates to the novel tumor antigen protein and the gene thereof, tumor antigen peptides derived from the tumor antigen protein, and derivatives of their substances, as well as to medicaments, or prophylactics that utilize *in vivo* or *in vitro* such tumor antigen protein, genes, tumor antigen peptides, or derivatives thereof.

[0002]

Prior art:

It is known that immune system, particularly T cells, plays an important role in tumor elimination by a living body. Indeed, infiltration of lymphocytes exhibiting cytotoxic effects on tumor cells in human tumor foci has been observed (*Arch. Surg.*, 126:200, 1990), and cytotoxic T lymphocytes (CTLs) recognizing autologous tumor cells have been isolated from melanomas without great difficulties (e.g., *Immunol. Today*, 8:385, 1987; *J. Immunol.*, 138:989, 1987; and *Int. J. Cancer*, 52:52, 1992). In addition, the results of clinical treatment of melanomas by transfer of the CTLs also suggest the importance of T cells in tumor elimination (*J. Natl. Cancer. Inst.*, 86:1159, 1994).

[0003]

Although it had long been unknown about target molecules for CTLs attacking autologous tumor cells, the recent

advance in immunology and molecular biology gradually began elucidating such target molecules. Specifically, it has been found that CTL, using the T cell receptors (TCRs), recognizes a complex between a peptide, called tumor antigen peptide, and a major histocompatibility complex class I antigen (MHC class I antigen, and in the case of human, referred to as HLA antigen), and thereby attacks autologous tumor cells.

[0004]

Tumor antigen peptides are generated by degradation of tumor antigen proteins, which are proteins specific for tumors, in cells with proteasomes, which proteins are intracellularly synthesized. The tumor antigen peptides thus generated bind to MHC class I antigens (HLA antigens) in endoplasmic reticulum to form complexes, and the complexes are transported to the cell surface to be presented as an antigen. A tumor-specific CTL recognizes the complex presented as an antigen, and exhibits anti-tumor effects through its cytotoxic action or production of lymphokines. As a consequence of elucidation of a series of the actions, it has become possible to treat tumors by using tumor antigen proteins or tumor antigen peptides as so-called cancer vaccines to enhance tumor-specific CTLs in the body of a tumor patient.

[0005]

As a tumor antigen protein, T. Boon *et al.* identified a protein named MAGE from human melanoma cells for the first time in 1991 (*Science*, 254:1643, 1991). Subsequently, several additional tumor antigen proteins have been identified

mainly from melanoma cells. Examples of melanoma antigens that have been identified are melanosomal proteins such as a melanocytic tissue-specific protein, gp100 (*J. Exp. Med.*, 179:1005, 1994), MART-1 (*Proc. Natl. Acad. Sci. USA*, 91:3515, 1994), and tyrosinase (*J. Exp. Med.*, 178:489, 1993); MEGE-related proteins that are expressed not only on melanomas but also on various cancer cells and normal testicular cells (*J. Exp. Med.*, 179:921, 1994); β-catenin having a tumor-specific amino acid mutation (*J. Exp. Med.*, 183:1185, 1996); and CDK4 (*Science*, 269:1281, 1995). Tumor antigen proteins other than those from melanomas have also been identified, including products of oncogenes such as HER2-neu (*J. Exp. Med.*, 181:2109, 1995) and p53 (variant) (*Proc. Natl. Acad. Sci. USA*, 93:14704, 1996); tumor markers such as CEA (*J. Natl. Cancer Inst.*, 87:982, 1995) and PSA (*J. Natl. Cancer Inst.*, 89:293, 1997); and viral proteins such as HPV (*J. Immunol.*, 154:5934, 1995) and EBV (*Int. Immunol.*, 7:653, 1995). Detailed descriptions of these substances can be found in published reviews (e.g. *Immunol. Today*, 18:267, 1997; *J. Exp. Med.*, 183:725, 1996; and *Curr. Opin. Immunol.*, 8:628, 1996).

[0006]

In applications of a tumor antigen protein or a tumor antigen peptide to treatment or diagnosis of tumors, it is important to identify a tumor antigen that can be widely applied to squamous cell carcinomas such as esophageal and lung cancers that occur at a much higher incidence compared to melanomas. In this relation, the present inventors conducted

cloning of a gene encoding a novel tumor antigen protein from squamous cell carcinoma cells derived from esophageal cancer, and identified for the first time from the tumor cell other than melanomas several tumor antigen peptides that are bound to and presented on HLA antigens of which HLA types are HLA-A24 or HLA-A26 (*J. Exp. Med.*, 187:277, 1998; International Patent Publication WO 97/46676).

[0007]

When these tumor antigen peptides are clinically applied in practice, it may be desirable to use two or more different tumor antigen peptides rather than to use merely one peptide. That is to say, taking into consideration the facts that all cancer cells do not express an identical tumor antigen in common and that two or more different tumor antigen peptides are presented on a single cancer cell, a treatment using two or more different tumor antigen peptides is believed to be more effective. Indeed, in the case of melanoma, development of cocktail formulations comprising two or more peptides has been attempted, since a single peptide derived from a tumor antigen failed to exhibit adequate effects (*Int. J. Cancer*, 66:162, 1996; and *Int. J. Cancer*, 67:54, 1996). Under such circumstances, it is being required to identify novel tumor antigen proteins and tumor antigen peptides that can be widely applied to squamous cell carcinomas that occur at a higher incidence.

[0008]

Problem to be solved by the invention:

The present invention aims to provide the novel tumor antigen protein and tumor antigen peptides. Particularly, it aims to provide the novel tumor antigen protein and gene thereof, tumor antigen peptides derived from the tumor antigen protein, and derivatives of their substances, as well as to provide medicaments, or prophylactics for tumors that utilize *in vivo* or *in vitro* such tumor antigen protein, genes, tumor antigen peptides, or derivatives thereof. The tumor antigen peptides of the present invention include a tumor antigen peptide that is bound to and presented on HLA-A24 that is the HLA antigen carried by about 60% of the Japanese people, and, therefore, it can be applied to many patients. Further, the tumor antigen peptides of the present invention may also be applied to squamous cell carcinomas or the like that is recognized most frequently as an etiologic cancer in human, and are expected to have utilities as novel anti-tumor medicaments. It is known that the squamous cell carcinoma on esophageal or lung cancer among the squamous cell carcinomas tends to relatively exhibit a resistance to the current chemotherapy and radiotherapy. In this respect, the development of the tumor antigen peptides of the present invention is desired.

[0009]

In order to obtain novel tumor antigen protein and tumor antigen peptides, the present inventors made the following attempts.

First of all, the present inventors prepared a cDNA library from esophageal cancer cell line KE-4 (FERM BP-5955),

and doubly transfected fibroblast cell line VA-13 (RIKEN CELL BANK, The Institute of Physical and Chemical Research) with a recombinant plasmid of the library and a recombinant plasmid containing cDNA of HLA-A2402 (one type of HLA-A24). The resulting transfectants were treated with KE-4CTL (FERM BP-5954) that is directed to KE-4, and the amount of produced IFN-γ was measured to determine whether or not KE-4CTL was activated. As a result of such extensive screening repeatedly conducted, the present inventors finally succeeded in cloning one gene encoding a tumor antigen protein. The inventors named the tumor antigen protein encoded by the gene "SART-3". Comparing the base sequence of SART-3 with known sequences revealed that said base sequence of SART-3 was a novel base sequence that is different from the KIAA0156 gene registered as Accession No. D63879 at GenBank database in terms of a single base, which function has not been demonstrated.

[0010]

Further, the present inventors identified tumor antigen peptide portions residing in the amino acid sequence of SART-3 that are bound to and presented on HLA-A24, and demonstrated that such peptides have activity as a tumor antigen peptide.

The present invention has been completed on the basis of the findings as described above.

[0011]

Thus, the present invention relates to:

(1) A DNA encoding a protein having an amino acid

sequence shown in SEQ ID NO: 1, or a protein variant having an amino acid sequence containing substitution, deletion, and/or addition of one or more amino acid residues of SEQ ID NO: 1, provided that the protein and the protein variant give rise to tumor antigen peptides that are capable of binding to an HLA antigen and being recognized by cytotoxic T lymphocytes;

(2) A DNA consisting of a base sequence shown in SEQ ID NO: 2, or a DNA variant that hybridizes to the DNA under a stringent condition, provided that a protein produced and expressed by the DNA or the DNA variant gives rise to tumor antigen peptides that are capable of binding to an HLA antigen and being recognized by cytotoxic T lymphocytes;

(3) An expression plasmid that contains the DNA of the above (1) or (2);

(4) A transformant that is transformed with the expression plasmid of the above (3);

(5) A process for producing a recombinant protein, which comprises culturing the transformant of the above (4), and recovering the expressed recombinant protein;

(6) A tumor antigen protein that is produced by expressing the DNA of the above (1) or (2);

(7) A pharmaceutical composition that comprises as an active ingredient the DNA of the above (1) or (2), or the protein of the above (6);

(8) A pharmaceutical composition for treating or preventing tumors, which comprises as an active ingredient the DNA of the above (1) or (2), or the protein of the above (6);

(9) A tumor antigen peptide that is a partial peptide derived from the protein of the above (6), and that is capable of binding to an HLA antigen and being recognized by cytotoxic T lymphocytes, or a derivative thereof having the functionally equivalent properties;

(10) The tumor antigen peptide of the above (9) wherein the HLA antigen is HLA-A24 or HLA-A2, or a derivative thereof having the functionally equivalent properties;

(11) The tumor antigen peptide of the above (10), which is selected from those sequences, each comprising all or part of an amino acid sequence shown in any one of SEQ ID NOs: 3-24, or a derivative thereof having the functionally equivalent properties;

(12) The tumor antigen peptide of the above (11), which comprises all or part of an amino acid sequence shown in any one of SEQ ID NOs: 3-9, or a derivative thereof having the functionally equivalent properties;

(13) The tumor antigen peptide derivative of the above (12), which comprises all or part of an amino acid sequence wherein the amino acid residue at position 2 and/or the C-terminus in the amino acid sequence shown in any one of SEQ ID NOs: 3-9 is substituted by another amino acid residue;

(14) The tumor antigen peptide derivative of the above (13), which comprises all or part of the amino acid sequence shown in any one of SEQ ID NOs: 25-31;

(15) A pharmaceutical composition for treating or preventing tumors, which comprises as an active ingredient at

least one substance selected from the tumor antigen peptides and derivatives thereof according to any one of the above (9) to (14);

(16) An antibody that specifically binds to any one of the protein of the above (6), and the tumor antigen peptide or the derivative thereof according to any one of the above (9) to (14);

(17) An antigen-presenting cell wherein a complex between an HLA antigen and the tumor antigen peptide or the derivative thereof according to any one of the above (9) to (14) is presented on the surface of a cell having antigen-presenting ability, which cell is isolated from a tumor patient;

(18) A pharmaceutical composition for treating tumors, which comprises as an active ingredient the antigen-presenting cell of the above (17);

(19) A cytotoxic T lymphocyte that specifically recognizes a complex between an HLA antigen and the tumor antigen peptide or derivative thereof according to any one of the above (9) to (14); and

(20) A pharmaceutical composition for treating tumors, which comprises as an active ingredient the cytotoxic T lymphocyte of the above (19).

[0012]

Mode for carrying out the invention:

The DNAs of the present invention encode novel tumor antigen proteins, and specific examples of the DNAs include a DNA encoding SART-3 protein having an amino acid

sequence shown in SEQ ID NO: 1, or a protein variant having an amino acid sequence containing substitution, deletion, and/or addition of one or more amino acid residues of the amino acid sequence of SART-3, provided that the protein and the protein variant give rise to tumor antigen peptides that are capable of binding to an HLA antigen and being recognized by cytotoxic T lymphocytes; or a DNA of SART-3 consisting of a base sequence shown in SEQ ID NO: 2, or a DNA variant that hybridizes to the DNA of SART-3 under a stringent condition, provided that a protein produced and expressed by the DNA and the DNA variant gives rise to tumor antigen peptides that are capable of binding to an HLA antigen and being recognized by cytotoxic T lymphocytes. The DNA of the present invention is further described hereinafter following the order established above.

[0013]

1) DNA encoding SART-3

"A DNA encoding a protein having an amino acid sequence shown in SEQ ID NO: 1" and "a DNA consisting of a base sequence shown in SEQ ID NO: 2" among the DNAs described above refers to a DNA encoding tumor antigen protein SART-3 of the present invention. The DNA may be cloned in accordance with the process described in Examples hereinafter. Further, the cloning of the DNA may also be conducted by, for example, screening a cDNA library derived from cell lines such as esophageal cancer cell line KE-4 (FERM BP-5955) using an appropriate portion of the base sequence disclosed in GenBank Accession No. D63879 or shown in SEQ ID NO: 2 in the present

specification as a probe for hybridization or a PCR primer. It would be ready for those skilled in the art to achieve such cloning in accordance with Molecular Cloning 2nd Edt. Cold Spring Harbor Laboratory Press (1989), for example.

[0014]

2) DNA encoding a modified protein of SART-3 or allelic variant thereof

"DNA encoding a protein variant having an amino acid sequence containing substitution, deletion, and/or addition of one or more amino acid residues of the amino acid sequence of SART-3" among the DNAs described above refers to a DNA that encodes a so-called modified protein, which is artificially prepared, or proteins such as an allelic variant existing in a living body. The DNA encoding such protein variants may be prepared by diverse methods such as site-directed mutagenesis and PCR technique that are described in Molecular Cloning: A Laboratory Manual 2nd Edt. vols. 1-3, Cold Spring Harbor Laboratory Press (1989). The number of amino acid residues to be substituted, deleted and/or added should be in a range that enables the substitution, deletion, and/or addition in accordance with the well-known methods such as site-directed mutagenesis as shown above.

[0015]

3) DNA that hybridizes to the DNA of SART-3 under a stringent condition

"DNA variant that hybridizes to the DNA of SART-3 under a stringent condition" among the DNAs described above

refers to a DNA that hybridizes to human SART-3 cDNA consisting of the base sequence shown in SEQ ID NO: 2 under a stringent condition, including SART-3 DNAs from all of vertebrate such as rat and mouse.

[0016]

The term "stringent condition" refers to a condition such that a hybridization is conducted in a solution containing 6xSSC (20xSSC represents 333 mM Sodium citrate, 333 mM NaCl), 0.5% SDS and 50% formamide at 42°C, and then the hybridized products are washed in a solution of 0.1xSSC, 0.5%SDS at 68°C, or to conditions as described in Nakayama, et al., *Bio-Jikken-Illustrated*, vol. 2, "Idenshi-Kaiseki-No-Kiso (A Basis for Gene Analysis)", pp. 148-151, Shujunsha, 1995.

[0017]

The DNA variants are cloned by diverse processes such as hybridization to the DNA shown in SEQ ID NO: 2. Particular procedures for the processes such as production of cDNA library, hybridization, selection of positive colony, and determination of base sequence are well-known, and may be conducted consulting Molecular Cloning as shown above. Probes useful for the hybridization includes a DNA comprising a base sequence described in SEQ ID NO: 2.

[0018]

Among the DNAs as described above 1) to 3), a DNA having an ability to generate a tumor antigen peptide that is capable of binding to an HLA antigen and being recognized by CTLs, and that is derived from a protein produced by the

expression of the DNA via intracellular degradation, constitutes the DNA encoding tumor antigen protein of the present invention, namely , the DNA of the present invention. Particularly, the DNAs of the present invention may be those that generate such peptide fragment as a partial peptide consisting of a part of an amino acid sequence of a protein produced by the expression of said DNA, said peptide being capable of binding to an HLA antigen, and inducing production of cytotoxic actions and cytokines from CTLs specific for the complex between the peptide and the HLA antigen that bind to the complex presenting on the cell surface.

[0019]

Determination whether or not a candidate DNA may be a DNA encoding a tumor antigen protein may be achieved for example by the following method.

An expression plasmid containing a candidate DNA and an expression plasmid containing a DNA encoding an HLA antigen are doubly transfected into fibroblast VA-13 (RIKEN CELL BANK, The Institute of Physical and Chemical Research) or COS-7 (ATCC CRL 1651) derived from African green monkey kidney. The transfection may be achieved, for example, by the Lipofectin method using Lipofectamine reagent (GIBCO BRL). Subsequently, a tumor-responsive CTL that is restricted to the particular HLA antigen used is added to act on the transfectants, and then the amount of various cytokines (for example, IFN-γ) produced by said CTL in response to the transfectants may be measured. In this context, since SART-3 contains HLA-A24-restricted tumor

antigen peptide portions, HLA-A24 cDNA (*Cancer Res.*, 55:4248-4252 (1995); Genbank Accession No. M64740) may be used as the above DNA encoding the HLA antigen, whereas those CTLs that are HLA-A24-restricted CTLs such as KE-4CTL (FERM BP-5954) may be used as the above CTL.

[0020]

The DNA of the present invention as described above can be used as an active ingredient in a medicament or a pharmaceutical composition. That is, in a pharmaceutical composition that comprises the DNA of the present invention as an active ingredient, administration of the DNA of the present invention to a tumor patient makes treatment or prevention of tumors possible.

[0021]

By administering a DNA of the present invention, the tumor antigen protein is highly expressed in antigen-presenting cells. Tumor antigen peptides bind to HLA antigen to form complexes, and the complexes are densely presented on the antigen-presenting cell surface. As a result, CTLs specific for tumors efficiently proliferate in the body. In this way, treatment or prevention of tumors is achieved. Administration and introduction of the DNA of the present invention into cells may be achieved using viral vectors or according to any one of other procedures (*Nikkei-Science*, April, 1994, pp. 20-45; *Gekkan-Yakuji*, 36(1), 23-48 (1994); *Jikken-Igaku-Zokan*, 12(15), 1994, and references cited therein).

[0022]

Examples of the methods using viral vectors include methods in which a DNA of the present invention is incorporated into a DNA or RNA virus such as retrovirus, adenovirus, adeno-associated virus, herpesvirus, vaccinia virus, poxvirus, poliovirus, or Sindbis virus, and introduced into cells. Among these methods, those using retrovirus, adenovirus, adeno-associated virus, or vaccinia virus are particularly preferred.

Other methods include those in which expression plasmids are directly injected intramuscularly (DNA vaccination), liposome method, Lipofectin method, microinjection, calcium phosphate method, and electroporation, and DNA vaccination and liposome method are particularly preferred.

[0023]

In order to allow a DNA of the present invention to act as a medicament in practice, there are an *in vivo* method in which DNA is directly introduced into the body, and an *ex vivo* method in which certain cells are removed from human, and after introducing DNA into said cells extracorporeally, the cells are reintroduced into the body (*Nikkei-Science*, April, 1994, pp. 20-45; *Gekkan-Yakuji*, 36(1), 23-48 (1994); *Jikkenn-Igaku-Zokan*, 12(15), 1994; and references cited therein). An *in vivo* method is more preferred.

[0024]

In case of *in vivo* methods, the DNA may be administered by any appropriate route depending on the disease

and symptoms to be treated. For example, it may be administered via intravenous, intraarterial, subcutaneous, intracutaneous, intramuscular route, or the like. In the case of *in vivo* methods, the compositions may be administered in various dosage forms such as solution, and are typically formulated, for example, in the form of injection containing a DNA of the present invention as an active ingredient, to which conventional carriers may also be added, if necessary. If a DNA of the present invention is included in liposomes or membrane-fused liposomes (such as Sendai virus (HVJ)-liposomes), the compositions may be in the form of liposome formulations such as suspension, frozen drug, centrifugally-concentrated frozen drug, or the like.

Although the amount of a DNA of the present invention in such formulations may vary depending on the disease to be treated, the age and the weight of the patient, and the like, it is typical to administer 0.0001mg-100 mg, preferably 0.001mg-10 mg, of a DNA of the present invention every several days to every several months.

[0025]

In the invention, the term "protein" refers to a protein encoded by the various DNAs of the present invention as described above, which has an ability as tumor antigen protein to give rise to tumor antigen peptides via intracellular degradation that are capable of binding to an HLA antigen and being recognized by CTLs. Specific examples of the proteins include SART-3 comprising an amino acid sequence shown in SEQ

ID NO: 1. The proteins of the present invention may be produced in large scale using the DNA of the present invention as described above.

[0026]

Production of tumor antigen proteins by expressing the DNA of the present invention may be achieved in accordance with many publications and references such as "Molecular Cloning" mentioned above. Particularly, an expression plasmid that replicates and functions in host cells is constructed by incorporating a DNA of the present invention into an appropriate expression vector (e.g., pSV-SPORT1, pCR3). Subsequently, the expression plasmid is introduced into appropriate host cells to obtain transformants. Examples of host cells include those of prokaryotes such as *Escherichia coli*, unicellular eukaryotes such as yeast, and cells derived from multicellular eukaryotes such as insects or animals. Gene transfer into host cells may be achieved by conventional methods such as calcium phosphate method, DEAE-dextran method, electric pulse method, Lipofectin method, or the like. Desired proteins are produced by culturing the transformants in appropriate medium. The tumor antigen proteins thus obtained may be isolated and purified according to standard biochemical procedures.

[0027]

The protein of the present invention as described above can also be used as an active ingredient in medicament or a pharmaceutical composition. That is, in a "pharmaceutical composition" that comprises the protein of the present

invention as an active ingredient, administration of the protein to a tumor patient makes treatment or prevention of tumors possible.

Pharmaceutical compositions comprising the tumor antigen protein of the present invention as an active ingredient may be administered together with an adjuvant in order to effectively establish the cellular immunity, or may be administered in a particulate dosage form. For such purpose, those adjuvants described in the literature (*Clin. Microbiol. Rev.*, 7:277-289, 1994) are applicable. In addition, liposomal preparations, particulate preparations in which the ingredient is bound to beads having a diameter of several μm, or preparations in which the ingredient is attached to lipids are also possible. Administration may be achieved, for example, intradermally, hypodermically, or by intravenous injection. Although the amount of a tumor antigen protein of the present invention in such formulations may vary depending on the disease to be treated, the age and the weight of the patient, and the like, it is typical to administer 0.0001mg-1000 mg, preferably 0.001mg-1000 mg, more preferably 0.1mg-10mg of a tumor antigen protein of the present invention every several days to every several months.

[0028]

In the present invention, the term "tumor antigen peptide" refers to a partial peptide that consists of a part of the tumor antigen protein of the present invention and is capable of binding to an HLA antigen and being recognized by

CTL. Accordingly, any peptide falls within the scope of the tumor antigen peptide of the present invention, regardless of its length or its position in the amino acid sequence of the present protein, as long as the peptide consists of a part of the amino acid sequence of the present protein and a complex between said peptide and an HLA antigen is capable of being recognized by CTL. Such tumor antigen peptides of the present invention can be identified by synthesizing a candidate peptide which consists of a part of the tumor antigen protein of the present invention and conducting an assay for determining whether or not a complex between the candidate peptide and an HLA antigen is recognized by CTL, in other words, whether or not the candidate peptide has the activity as a tumor antigen peptide.

[0029]

In this connection, synthesis of peptides may be conducted according to a method usually used in peptide chemistry. Examples of such known methods are those described in the literatures including "Peptide Synthesis", Interscience, New York, 1966; "The Proteins", vol. 2, Academic Press Inc., New York, 1976; "Pepuchido-Gosei", Maruzen Co. Ltd., 1975; "Pepuchido-Gosei-no-Kiso-to-Jikkenn", Maruzen Co. Ltd., 1985; and "Iyakuhin-no-Kaihatu, Zoku, vol. 14, Peputido-Gosei", Hirokawa Shoten, 1991.

[0030]

Next, methods for identifying tumor antigen peptides of the present invention are further described below.

The respective sequence rules (motifs) of antigen peptides that are bound to and presented on the following HLA types have been known; HLA-A1, -A0201, -A0204, -A0205, -A0206, -A0207, -A11, -A24, -A31, -A6801, -B7, -B8, -B2705, -B37, -Cw0401, and -Cw0602 (see, e.g., *Immunogenetics*, 41:178, 1995). Regarding the motif for HLA-A24, for example, it is known that in the sequence of peptides consisting of 8 to 11 amino acids, the amino acid at position 2 is tyrosine, phenylalanine, methionine, or tryptophan, and the amino acid at the C-terminus is phenylalanine, leucine, isoleucine, tryptophan, or methionine (*J. Immunol.*, 152:3913, 1994). Likewise, the motifs shown in the following Table 1 are known for HLA-A2 (*Immunogenetics*, 41:178, 1995; *J. Immunol.*, 155:4749, 1995).

[0031]

[Table 1]

Type of HLA-A2	position from N-terminus	Amino acid at C-terminus
HLA-A0201	L, M	V, L
HLA-A0204	L	L
HLA-A0205	V, L, I, M	L
HLA-A0206	V, Q	V, L
HLA-A0207	L	L

(the peptides are 8-11 amino acids in length)

[0032]

By analysis of antigen peptides bound to various HLA molecules, it has been shown that the length of the peptides is usually about 8 to 14 amino acids long, although antigen

peptides of 14 or more amino acids in length are also observed for HLA-DR, -DP, and -DQ (*Immunogenetics*, 41:178, 1995).

[0033]

It is easy to select peptide portions involved in such motifs from the amino acid sequence of the protein of the present invention. For example, such peptide portions involved in the above motif structures can be easily selected by inspecting the amino acid sequence of tumor antigen protein SART-3 (SEQ ID NO: 1). Tumor antigen peptides of the present invention can be identified by synthesizing candidate peptides thus selected according to the method described above and conducting an assay for determining whether or not a complex between the candidate peptide and an HLA antigen is recognized by CTL, in other words, whether or not a candidate peptide has an activity as a tumor antigen peptide.

[0034]

A specific example of method for identifying tumor antigen peptides of the present invention is a method described in *J. Immunol.*, 154:2257,1995. Specifically, peripheral blood lymphocytes are isolated from a human who is positive for the type of an HLA antigen that is expected to present the candidate peptide, and are stimulated *in vitro* by adding the candidate peptide. If the candidate induces CTL that specifically recognizes the HLA-antigen-presenting cells pulsed with the candidate peptide, it is indicated that the particular candidate peptide may function as a tumor antigen peptide. In this connection, the presence or absence of CTL induction can

be detected, for example, by measuring the amount of various cytokines (for example, IFN-γ) produced by CTLs in response to the antigen peptide-presenting cells. Alternatively, a method in which the cytotoxicity of CTLs against antigen peptide-presenting cells labeled with ^{51}Cr is measured (^{51}Cr release assay, *Int. J. Cancer*, 58:317, 1994) may also be used for such detection.

Furthermore, the above detection can also be achieved as follows. An expression plasmid expressing a cDNA for the type of an HLA antigen that is expected to present the candidate peptide is incorporated into, for example, COS-7 cells (ATCC No. CRL1651) or VA-13 cells (RIKEN CELL BANK, The Institute of Physical and Chemical Research), and the resultant cells are pulsed with the candidate peptide. The cells are then reacted with the CTLs that are restricted to the type of the HLA antigen expected to present the candidate peptide as described above, and the amount of various cytokines (for example, IFN-γ) produced by said CTLs is measured (*J. Exp. Med.*, 187:277, 1998).

[0035]

In cases like HLA-A26 wherein a relevant peptide motif is not elucidated, tumor antigen peptides of the present invention can be identified, for example, according to the method described in WO 97/46676, which method is different from that in the above cases wherein the sequence rules (motifs) have been elucidated, provided that a CTL line recognizing a complex between the HLA-A26 and a tumor antigen peptide is available.

The methods for identifying tumor antigen peptides as described above may be hereinafter collectively referred to as "assay methods for tumor antigen peptides".

[0036]

As described above, it is known that the sequences of tumor antigen peptides that are bound to and presented on HLA-A24 obey a certain rule (motif), and in particular, the motif is that, in a sequence of a peptide consisting of 8 to 11 amino acids, the amino acid at position 2 is tyrosine, phenylalanine, methionine, or tryptophan, and the amino acid at the C-terminus is phenylalanine, leucine, isoleucine, tryptophan, or methionine (*J. Immunol.*, 152:3913, 1994). Accordingly, HLA-A24-restricted tumor antigen peptides among the tumor antigen peptides of the present invention are exemplified by those tumor antigen peptides that are partial peptides involved in such motif structures or structures expected to be capable of binding to the HLA-A24-antigen in the amino acid sequence of SART-3 shown in SEQ ID NO: 1 and that are capable of binding to the HLA-24-antigen and being recognized by CTLs.

[0037]

For example, those tumor antigen peptides that each comprise all or part of the amino acid sequence shown in any one of SEQ ID NOs: 3-24 and that are capable of binding to an HLA-A24 antigen and being recognized by CTL are exemplified.

Specifically, the following tumor antigen peptides are given:

1) a peptide consisting of the amino acid sequence shown in any one of SEQ ID NOs: 3-24, and

2) a peptide comprising the amino acid sequence shown in any one of SEQ ID NOs: 3-24, and being longer than said amino acid sequence, or a peptide comprising a partial amino acid sequence of the sequence shown in any one of SEQ ID NOs: 3-24,

said peptide being capable of binding to an HLA-24-antigen and being recognized by CTL. The peptide in the above 2) may be about 8-11 amino acids in length in view of the fact that they are bound and presented by an HLA-24-antigen.

[0038]

Suitable examples of HLA-A24-restricted tumor antigen peptides of the present invention include those tumor antigen peptides that each comprise all or part of the amino acid sequence shown in any one of SEQ ID NOs: 3-9 and that are capable of binding to an HLA-A24 antigen and being recognized by CTL. Specifically, the following tumor antigen peptides are given:

1) a peptide that consists of an amino acid sequence shown in any one of SEQ ID NOs: 3-9, and

2) a peptide that comprises an amino acid sequence shown in any one of SEQ ID NOs: 3-9 and that is longer than said amino acid sequence, or a peptide that comprises a partial amino acid sequence of the sequence shown in any one of SEQ ID NOs: 3-24,

said peptide being capable of binding to an HLA-A24 antigen and being recognized by CTL. The peptides in the above 2) may be

about 8-11 amino acids in length in view of the fact that they are bound to and presented on HLA-A24 antigens.

[0039]

In the present invention, the term "derivative having properties functionally equivalent to those of a tumor antigen peptide" (hereinafter may be simply referred to as tumor antigen peptide derivative) refers to an altered peptide, of which amino acid sequence contains alteration of one or more, preferably one to several, amino acid residues of an amino acid sequence of a tumor antigen peptide of the present invention, and which has the properties as a tumor antigen peptide, that are to be capable of binding to an HLA antigen and being recognized by CTL. Such tumor antigen peptide derivatives of the present invention may be identified by synthesizing altered peptides that contain alteration of a part of a tumor antigen peptide of the present invention in accordance with the above preparation of peptide, and by conducting the above assay for tumor antigen peptides.

[0040]

As described above, the sequence rules (motifs) for peptides that are bound to and presented on HLA types such as HLA-A1, -A0201, -A0204, -A0205, -A0206, -A0207, -A11, -A24, -A31, -A6801, -B7, -B8, -B2705, -B37, -Cw0401, and -Cw0602 have been elucidated. Consequently, tumor antigen peptide derivatives containing the alteration of the amino acids in a tumor antigen peptide of the present invention can be prepared on the basis of such motifs.

[0041]

For example, regarding the motif for antigen peptides that are bound to and presented on HLA-A24, it is known as described above that, in the sequence of a peptide consisting of 8 to 11 amino acids, the amino acid at position 2 is tyrosine, phenylalanine, methionine, or tryptophan, and the amino acid at the C-terminus is phenylalanine, leucine, isoleucine, tryptophan, or methionine (*J. Immunol.*, 152:3913, 1994). Likewise, the motifs shown in the above Table 1 are known for HLA-A2. Accordingly, examples of tumor antigen peptide derivatives of the present invention include those peptide derivatives, in each of which one or more amino acid residues at any positions that may be allowed for substitution according to the motifs (for HLA-A24 and HLA-A2, position 2 and the C-terminus) are substituted by other amino acids. Preferred examples are those tumor antigen peptide derivatives, in each of which amino acid residues to be substituted are selected from those at said positions according to the above motifs.

[0042]

Examples of HLA-A24-restricted tumor antigen peptide derivatives include those peptide derivatives that each comprise all or part of an amino acid sequence in which one or more amino acid residues at positions that are allowed for substitution according to the above motifs, specifically, at position 2 and/or the C-terminus, of a peptide derived from the amino acid sequence of SART-3 having a binding motif for HLA-A24 are substituted by other amino acid residues. Preferred

examples are those tumor antigen peptide derivatives that each comprise all or part of an amino acid sequence in which the amino acid residues at position 2 and/or the C-terminus are substituted by the amino acid residues involved according to the above motifs.

[0043]

Examples are those tumor antigen peptide derivatives that each comprise all or part of an amino acid sequence in which the amino acid residues at position 2 and/or the C-terminus of an amino acid sequence shown in any one of SEQ ID NOs: 3 to 24 are substituted by other amino acid residues. Particularly, those tumor antigen peptide derivatives that each comprise all or part of an amino acid sequence in which the amino acid residues at position 2 and/or the C-terminus of an amino acid sequence shown in any one of SEQ ID NOs: 3 to 24 are substituted by tyrosine, phenylalanine, methionine, or tryptophan and/or the amino acid residue at the C-terminus is substituted by phenylalanine, leucine, isoleucine, tryptophan, or methionine are exemplified.

[0044]

Suitable examples of HLA-A24-restricted tumor antigen peptide derivatives of the present invention are those tumor antigen peptide derivatives that each comprise all or part of an amino acid sequence in which the amino acid residues at position 2 and/or the C-terminus of the amino acid sequence shown in any one of SEQ ID NOs: 3 to 9 are substituted by other amino acid residues. More preferred examples are those tumor

antigen peptide derivatives that each comprise all or part of an amino acid sequence in which the amino acid residue at position 2 of an amino acid sequence shown in any one of SEQ ID NOs: 3 to 9 is substituted by tyrosine, phenylalanine, methionine, or tryptophan and/or the amino acid residue at the C-terminus is substituted by phenylalanine, leucine, isoleucine, tryptophan, or methionine. Suitable examples of such tumor antigen peptide derivatives are shown in SEQ ID NOs: 25 to 31.

[0045]

A tumor antigen peptide or its derivative of the present invention can be used solely or together with other one or more of them as a pharmaceutical composition for treating or preventing tumors. Namely, the present invention provides a pharmaceutical composition for treatment or prevention for tumors, which comprises the tumor antigen peptides or derivatives thereof as an active ingredient. When the composition for treating or preventing tumors which comprises as an active ingredient a tumor antigen peptide or its derivative of the present invention is administered to a SART-3-positive patient, the tumor antigen peptide or derivative thereof is presented with an HLA antigen of antigen-presenting cells, and therefore, CTLs specific for the presented HLA antigen complex proliferate and destroy the tumor cells. As a result, the tumor of the patient may be treated or prevented. SART-3 is developed extensively on the squamous cell carcinoma such as esophageal cancer, and therefore, the

composition for treating or preventing tumors according to the present invention is advantageous in terms of wide applicability. The squamous cell carcinoma often exhibits a resistance to chemotherapy and radiotherapy, and, therefore, the composition for treating tumors of the present invention can also achieve an increased therapeutic effect by its combined use.

[0046]

The composition for treating or preventing tumors comprising as an active ingredient a tumor antigen peptide or its derivative of the present invention may be administered together with an adjuvant in order to effectively establish the cellular immunity, or may be administered in a particulate dosage form. For such purpose, those adjuvants described in the literature (*Clin. Microbiol. Rev.*, 7:277-289, 1994) are applicable. In addition, liposomal preparations, particulate preparations in which the ingredient is bound to beads having a diameter of several μm, or preparations in which the ingredient is attached to lipids are also possible. Administration may be achieved, for example, intradermally, hypodermically, or by intravenous injection. Although the amount of a tumor antigen peptide or its derivative of the present invention in the formulation to be administered may be adjusted as appropriate depending on, for example, the disease to be treated, the age and the body weight of the particular patient, it is typical to administer 0.0001 mg to 1000 mg, preferably 0.001 mg to 1000 mg, and more preferably 0.1 mg to

10 mg every several days to every several months.

[0047]

The present invention also provides antibodies that specifically bind to a protein of the present invention. Such antibodies are easily prepared, for example, according to a method described in "Antibodies: A Laboratory Manual", Lane, H. D. *et al.* eds., Cold Spring Harbor Laboratory Press, New York, 1989. Specifically, antibodies that recognize a tumor antigen peptide or its derivative and antibodies that further neutralize its activity may easily be prepared using the tumor antigen peptide or derivative thereof to appropriately immunize an animal in the usual manner. Such antibodies may be used in affinity chromatography, immunological diagnosis, and the like. Immunological diagnosis may be selected as appropriate from immunoblotting, radioimmunoassay (RIA), enzyme-linked immunosorbent assay (ELISA), a fluorescent or luminescent assay, and the like.

[0048]

A tumor antigen peptide, derivative thereof, tumor antigen protein, or gene therefor of the present invention may also be used *in vitro* for treatment of tumor patients as follows.

On usage of a tumor antigen peptide, derivative thereof, tumor antigen protein, or gene therefor in treatment of tumors, it is important to establish an administration method which can efficiently induce specific CTLs in the body of a patient. As one of the means therefor, the present invention provides an antigen-presenting cell in which a complex between

an HLA antigen and a tumor antigen peptide or its derivative of the present invention is presented on the surface of a cell having antigen-presenting ability isolated from a tumor patient, and also provides a pharmaceutical composition for treating tumors, which comprises said antigen-presenting cell as an active ingredient.

[0049]

In this context, the "cell having antigen-presenting ability" is not limited to a specific cell so long as it is a cell expressing on its cell surface an HLA antigen allowing a tumor antigen peptide or its derivative of the present invention to be presented, and dendritic cells, which are reported to have especially a high antigen-presenting ability, are preferred.

In order to prepare antigen-presenting cells of the present invention, cells having an antigen-presenting ability are isolated from a tumor patient, and pulsed *ex vivo* with a tumor antigen peptide, a derivative thereof, or a tumor antigen protein of the present invention to present a complex between an HLA antigen and said tumor antigen peptide or derivative thereof (*Cancer Immunol. Immunother.*, 46:82, 1998). Further, by introducing a gene encoding a tumor antigen peptide of the present invention into the antigen-presenting cells, a complex between an HLA antigen and the above peptide or its derivative may be prepared.

[0050]

A pharmaceutical composition for treating tumors

which comprises the above antigen-presenting cells as an active ingredient preferably contains physiological saline, phosphate buffered saline (PBS), medium, or the like to stably maintain the antigen-presenting cells. It may be administered, for example, intravenously, subcutaneously, or intradermally. By reintroducing such composition for treating tumors which comprises antigen-presenting cells as an active ingredient into the body of the patient, specific CTLs are efficiently induced in SART-3-positive patient so as to achieve treatment of the tumor. It should be undisputed that the HLA types need be compatible between the patient and the peptide used, such that an HLA-A24-restricted tumor antigen peptide or a derivative thereof must be used with an HLA-A24-positive tumor patient.

[0051]

In addition, *in vitro* use of a tumor antigen peptide, a derivative thereof, a tumor antigen protein, or a gene therefor according to the present invention in the following adoptive immunotherapy may be provided as another example of their use.

For melanomas, it has been observed that an adoptive immunotherapy wherein tumor-infiltrating T cells taken from the patient himself/herself are cultured *ex vivo* in large quantities, and then returned into the patient, achieves a therapeutic effect (*J. Natl. Cancer. Inst.*, 86:1159, 1994). Likewise, in mouse melanoma, suppression of metastasis has been observed by *in vitro* stimulation of splenocytes with tumor antigen peptide TRP-2, thereby proliferating CTLs specific for

the tumor antigen peptide, and administering said CTLs into a melanoma-grafted mouse (*J. Exp. Med.*, 185:453, 1997). This resulted from *in vitro* proliferation of CTLs that specifically recognize the complex between an HLA antigen of antigen-presenting cells and the tumor antigen peptide. Accordingly, a method for treating tumors is believed to be useful, which comprises stimulating *in vitro* peripheral blood lymphocytes from a patient using a tumor antigen peptide, a derivative thereof, a tumor antigen protein, or a gene therefor according to the present invention to proliferate tumor-specific CTLs, and subsequently returning the CTLs into the patient.

[0052]

Thus, the present invention provides CTLs that specifically recognize a complex between the HLA antigen and the tumor antigen peptide or derivative thereof, and also provides a pharmaceutical composition for treating tumors which comprises said CTLs as an active ingredient. Such composition preferably contains physiological saline, phosphate buffered saline (PBS), medium, or the like to stably maintain CTLs. It may be administered, for example, intravenously, subcutaneously, or intradermally. By reintroducing the composition for treating tumors which comprises CTLs as an active ingredient into the body of the patient, the toxic effect of CTLs against the tumor cells is enhanced in SART-3-positive patient and thereby destroys the tumor cells to achieve treatment of the tumor.

[0053]

[Examples]

The present invention is further illustrated by the following examples, but is not limited by these examples in any respect.

[0054]

Reference 1

Establishment of Cytotoxic T Lymphocyte (CTL) Cell Line against Esophageal Cancer Cell Line

According to the description of Nakao *et al.*, *Cancer Res.*, 55:4248-4252 (1995), CTL against esophageal cancer cell line KE-4, which belongs to squamous cell carcinomas when classified on the basis of tissue type, was established from peripheral blood mononuclear cells of a patient, named KE-4CTL, and used in the following experiments. Esophageal cancer cell lines KE-4 and KE-4CTL have been deposited at The National Institute of Bioscience and Human Technology (1-1-3 Higashi, Tsukuba, Ibaraki, Japan) under International Deposition Nos. FERM BP-5955 and FERM BP-5954, respectively, both on May 23, 1997. Further, typing of HLA class I molecules of KE-4 was conducted according to the above-mentioned description of Nakao *et al.*, to find that they are HLA-A2402, -A2601, -B54, -B60, -Cw1, and -Cw3.

[0055]

Reference 2

Preparation of HLA-A2402 cDNA

According to the description of Nakao *et al.*, *Cancer Res.*, 55: 4248-4252 (1995), a recombinant plasmid was

prepared from KE-4 by incorporating cDNA for HLA-A2402 (Genbank Accession No.M64740) into an expression vector pCR3 (INVITROGEN).

[0056]

Reference 3

Preparation of cDNA Library derived from KE-4

Poly $(A)^+$ mRNA was prepared from KE-4 by isolation of total RNA fraction and purification on oligo (dT) column using mRNA Purification system (Pharmacia Biotech) according to the manufacturer's protocol. cDNAs having *Not* I adapter and *Sca* I adapter linked to each terminus were prepared from the mRNAs using SuperScript Plasmid System (GIBCO BRL) according to the manufacturer's protocol, and then ligated into the restriction sites *Not* I and *Sal* I of an expression vector, plasmid pSV-SPORT1 (GIBCO BRL), to yield recombinant plasmids. The recombinant plasmids were introduced into *E. coli.* ElectroMAX DH10B™ cells (GIBCO BRL) using electric pulses in Gene Pulser (Bio-Rad) under a condition of 25 μF and 2.5 kV. Transformants into which the recombinant plasmids had been introduced were selected in LB medium (1% bacto-trypton, 0.5% yeast extract, 0.5% NaCl, pH7.3) containing ampicillin (50 μg/ml).

[0057]

Example 1

Screening of Novel Tumor Antigen Protein Gene

The recombinant plasmid DNAs were recovered as follows, from pools of about 100 transformants described in

Reference 3. A hundred transformants were introduced and cultured in each well of 96-well U-bottomed microplate containing LB medium plus ampicillin (50 μg/ml). Part of the culture was then transferred to another 96-well U-bottomed microplate containing 0.25 ml of TYGPN medium per well (F.M. Ausubel *et al.*, *Current Protocols in Molecular Biology*, John Wiley & Sons, Inc.), and cultured at 37°C for 48 hours. The remaining cultures in LB medium on the microplate were stored in frozen. Preparation of recombinant plasmid DNAs from transformants cultured in TYGPN medium was achieved in the microplate by the alkaline lysis method (F.M. Ausubel *et al.*, *Current Protocols in Molecular Biology*, John Wiley & Sons, Inc.). The recombinant plasmid DNAs recovered by isopropanol precipitation were suspended in 50 μl of 10 mM Tris, 1 mM EDTA, pH 7.4, containing 20 ng/ml RNase.

[0058]

The recombinant plasmid for KE-4 cDNA and the recombinant plasmid for HLA-A2402 cDNA were doubly transfected into fibroblast cell line VA-13 cells (RIKEN CELL BANK, The Institute of Physical and Chemical Research; *Ann. Med. Exp. Biol. Fenn.*, 44:242-254, 1966) using the Lipofectin method as follows. Specifically, seven thousands VA-13 cells were placed into each well of 96-well flat-bottomed microplate, and incubated for 2 days in 100 μl of RPMI 1640 medium containing 10% FCS. Using Lipofectin reagent (GIBCO BRL), a 30 μl portion of mixture 70 μl consisting of 25 μl of the recombinant plasmid for KE-4 cDNA corresponding to about 100 transformants, 10 μl (200 ng) of the

recombinant plasmid for HLA-A2402 cDNA described in Reference 2, and 35 μl of about 35-fold diluted Lipofectin reagent was added to VA-13 cells, and allowed to doubly transfect them. Transfectants were prepared in duplicate. After 5 hours, the transfectants were added with 200 μl of culture medium containing 10% FCS, and further incubated at 37 °C for 72 hours. After removing the culture medium, 10,000 KE-4CTL cells were added to each well, and cultured at 37°C for 24 hours in 100 μl of culture medium containing 10% FCS and 25 U/ml IL-2. The culture medium was recovered, and the amount of IFN-γ in the culture was measured by ELISA as described below.

[0059]

Specifically, an anti-human IFN-γ mouse monoclonal antibody was adsorbed on wells of 96-well microplate as a solid-phased antibody, and after blocking non-specific bindings with bovine serum albumin, the antibody was allowed to bind to IFN-γ in the above-described sample. Anti-human IFN-γ rabbit polyclonal antibody as a detection antibody was then allowed to bind, and after binding to an anti-rabbit immunoglobulin goat antibody labeled with alkaline phosphatase, para-nitrophenyl phosphate was reacted as a chromogenic substrate. After quenching the reaction by adding an equal volume of 1N NaOH, absorbance at 405 nm was measured. The absorbance was compared with that obtained with standard IFN-γ to determine the amount of IFN-γ in the sample.

[0060]

Regarding the groups in which high production of

IFN-γ was observed, the corresponding frozen-stored pools of about 100 transformants containing recombinant plasmids for KE-4 cDNA were used in the following screening. Specifically, the pools of the transformants were plated on LB agar medium containing ampicillin (50 μg/ml) to obtain colonies. Two hundreds colonies for each group were cultured as described above so that a single kind of transformant is included in each well, thereby preparing recombinant plasmid DNAs for KE-4 cDNA. Then, VA-13 cells were doubly transfected with the recombinant plasmid for KE-4 cDNA and the recombinant plasmid for HLA-A2402 cDNA, followed by co-cultivation with KE-4CTL, and IFN-γ produced due to KE-4CTL reaction was quantitatively determined as described above so as to select positive plasmids. In this manner, a single KE-4 cDNA recombinant plasmid clone was selected and named clone 13. Additional analysis revealed that clone 13 was incorporated with about 1.2 kb cDNA. Furthermore, similar procedures were repeated with clone 13 to determine the amount of IFN-γ produced by KE-4CTL according to a similar method to that described above. The results are shown in Table 2.

[0061]

[Table 2]

Target cell	Amount of IFN-γ produced by KE-4CTL (pg/ml)
VA-13 + HLA-A2402	326
VA-13 + HLA-A2402 + clone 13	775

[0062]

When compared to VA-13 transfected with only

HLA-A2402, KE-4CTL reacted more strongly to VA-13 doubly transfected with HLA-A2402 and clone 13, and produced more IFN-γ. This result indicated that the protein encoded by clone 13 is a tumor antigen protein.

[0063]

Example 2

Cloning of Full-length cDNA clone encoding Tumor Antigen Protein

In order to determine the length of the full-length cDNA gene incorporated in clone 13 obtained in Example 1, Northern Hybridization was conducted as described blow.

First of all, RNAs were prepared from esophageal cancer cell line KE-4 using RNAzol B (TEL-TEST, Inc.). Five μg of RNA was denatured in the presence of formamide and formaldehyde, electrophoresed on agarose, and then transferred and fixed onto Hybond-N+ Nylon membrane (Amersham). The inserted sequence region of clone 13 was labeled with ^{32}P using Multiprime DNA labeling system (Amersham) to prepare a DNA probe. According to the known method (Nakayama *et al.*, *Bio-Jikken-Illustrated*, vol. 2, "Idenshi-Kaiseki-No-Kiso (A Basis for Gene Analysis)", pp. 148-151, Shujunsha, 1995), this probe was allowed to hybridize to RNAs on the membranes, and subjected to autoradiography to detect mRNA for cDNA incorporated in clone 13, indicating that the mRNA was about 3.8 kb in full length. Then, the full-length cDNA clone containing clone 13 as prepared above was cloned. KE-4-derived cDNA Library described in Reference 3 was plated on LB agar medium containing ampicillin

(50μg/ml) to obtain colonies. The colonies were then transferred to and fixed on Hybond-N+ nylon membrane (Amersham) according to the manufacturer's protocol. DNA probe in which the insertion sequence of clone 13 was labeled with ^{32}P was employed for hybridization and autoradiography under similar conditions to those mentioned above in order to select colonies representing positive transformants. Recombinant plasmids were then recovered from the many colonies selected, treated with restriction enzymes *Not* I and *Sal* I, and then electrophoresed on agarose to determine the length of incorporated cDNAs. A recombinant plasmid incorporating cDNA of about 3.8 kb was selected, and named clone K. VA-13 Cells were then doubly transfected with the recombinant plasmid clone K incorporating cDNA for the tumor antigen protein gene and another recombinant plasmid containing cDNA for HLA-A2402 as described above, and the cells were used as target cells. The amount of IFN-γ produced by the reaction of KE-4CTL was determined according to the method as described above. The results are shown in Table 3.

[0064]

[Table 3]

Target cell	Amount of IFN-γ produced by KE-4CTL (pg/ml)
VA-13 + HLA-A2403	342
VA-13 + HLA-A2402 + clone K	627

[0065]

When compared to VA-13 transfected with only HLA-A2402, KE-4CTL reacted more strongly to VA-13 doubly

transfected with HLA-A2402 and clone K, and produced more IFN-γ. This result indicated that the protein encoded by clone K is a tumor antigen protein. The tumor antigen protein encoded by clone K is named SART-3 (squamous cell carcinoma antigens recognized by T cells - 3).

[0066]

Example 3

Determination of Base Sequence of Tumor Antigen Protein Gene

The base sequence of the DNA of tumor antigen protein SART-3 as obtained in Example 2 was determined using DyeDeoxy Terminator Cycle Sequencing kit (Perkin-Elmer). The base sequence thus determined is shown in SEQ ID NO: 2. The full-length of the cDNA was 3798 base pairs. The amino acid sequence (963 amino acids) encoded by the base sequence of SEQ ID NO: 2 is shown in SEQ ID NO: 1. Comparison of the base sequence shown in SEQ ID NO: 2 to known sequences using GenBank data base revealed that the base sequence of tumor antigen protein SART-3 has a novel base sequence that is different from gene KIAA0156 registered at GenBank under Accession No. D63879 in terms of a single base (at position 108 of KIAA0156), which function has not been demonstrated.

[0067]

Example 4

Selection of Candidate Peptides

There are certain rules (motifs) in the sequences of antigen peptides that should be bound and presented by HLA antigens. Regarding the motif for HLA-A24, it is known that

in the sequence of peptides consisting of 8 to 11 amino acids, the amino acid at position 2 is tyrosine, phenylalanine, methionine, or tryptophan, and the amino acid at the C-terminus is phenylalanine, tryptophan, leucine, isoleucine, or methionine (*Immunogenetics*, 41:178, 1995; *J. Immunol.*, 152:3913, 1994; *J. Immunol.*, 155:4307, 1994). According to the motifs, peptide portions consisting of 8 to 11 peptides having the above motifs were selected from the amino acid sequence of tumor antigen protein SART-3 shown in SEQ ID NO: 1. Those examples of the selected peptides are shown in SEQ ID NOs: 3-24. These peptides were synthesized at Biologica Co. by the Fmoc method.

[0068]

Then, 1.8×10^4 VA-13 cells were transfected with a recombinant plasmid of HLA-A2402 cDNA by the Lipofectin method to express HLA-A2402 according to the literature (*J. Exp. Med.*, 187:277, 1998). To these cells, various peptides having a binding motif for HLA-A24 that had precedently synthesized were each added at 10 μM over two hours in order to pulse the cells. The cells were then cultured with 2×10^4 KE-4CTLs for 18 hours, and the amount of IFN-γ produced by KE-4CTL in the culture supernatant was determined by the ELISA method. The results of this determination are shown in Table 4, which performed on seven peptides, that is, a peptide "109-118" comprising the sequence from position 109 to position 118 (SEQ ID NO: 3), a peptide "172-181" comprising the sequence from position 172 to position 181 (SEQ ID NO: 4), a peptide "284-292" comprising the

sequence from position 284 to position 292 (SEQ ID NO: 5), a peptide "315-323" comprising the sequence from position 315 to position 323 (SEQ ID NO: 6), a peptide "416-425" comprising the sequence from position 416 to position 425 (SEQ ID NO: 7), a peptide "426-434" comprising the sequence from position 426 to position 434 (SEQ ID NO: 8), and a peptide "448-456" comprising the sequence from position 448 to position 456 (SEQ ID NO: 9), in the amino acid sequence of tumor antigen protein SART-3.

[0069]

[Table 4]

Peptides	IFN-γ in the supernatant (pg/ml)
"109-118"	928
"172-181"	830
"284-292"	794
"315-323"	880
"416-425"	731
"426-434"	833
"448-456"	754
None	677

[0070]

When compared to cells pulsed with no peptide, KE-4CTLs reacted more strongly to cells pulsed with the peptides, and produced more IFN-γ. This result indicated that the seven peptides function as tumor antigen peptides.

[0071]

Example 5

Synthesis of Tumor Antigen Peptides

The seven peptides described above were synthesized by the solid phase method as shown below.

[0072]

(1) Synthesis of SART-3 "109-118" Val-Tyr-Asp-Tyr-Asn-Cys-His-Val-Asp-Leu (SEQ ID NO: 3)

Fmoc-Leu-Alko Resin (0.55 mmol/g, 100-200 mesh) was used as a resin. Using 100 mg of this resin, the synthesis was started according to Schedule 1 described below to couple the following residues in order: Fmoc-Asp(OtBu)-OH, Fmoc-Val-OH, Fmoc-His(Boc)-OH, Fmoc-Cys(Trt)-OH, Fmoc-Asn-OH, Fmoc-Tyr(tBu)-OH, Fmoc-Asp(OtBu)-OH, Fmoc-Tyr(tBu)-OH, and Fmoc-Val-OH. After the coupling, the procedures were conducted up to Step 3 of Schedule 1 to obtain a peptide resin.

[0073]

To this peptide resin, 2 ml of Reagent K (the solution of 5% phenol, 5% thioanisole, 5% H_2O, and 2.5% ethanedithiol in TFA) was added and the mixture was allowed to react for 2.5 hours at room temperature. While cooling with ice, 10 ml of diethyl ether was added to the reaction, the mixture was stirred for 10 minutes, filtered, and washed with 10 ml of diethyl ether. To the filter cake, 10 ml of aqueous acetic acid was added, and the mixture was stirred for 30 minutes. The resin was then filtered, and washed with 4 ml of aqueous acetic acid. After lyophilizing the filtrate and the wash, the crude peptide obtained was dissolved in aqueous acetic acid, and injected into a reverse phase packing material, YMC-PACK ODS-A column (30 ϕ x 250 mm) that had been pre-equilibrated with 0.1% aqueous TFA.

The column was washed with 0.1% aqueous TFA, and elution at a flow rate of 7 ml/min was then conducted, while increasing the concentration of acetonitrile up to 25% over 180 minutes. The eluate was monitored by A 220 nm. The fractions containing the desired product were combined together and lyophilized to obtain 31.0 mg of Val-Tyr-Asp-Tyr-Asn-Cys-His-Val-Asp-Leu.

[0074]

The peptide obtained, Val-Tyr-Asp-Tyr-Asn-Cys-His-Val-Asp-Leu, had a retention time of 19.3 minutes in an analysis using a reverse phase packing material, YMC-PACK ODS-AM column (4.6 ϕ x 250 mm) eluted with a linear gradient of acetonitrile concentration from 16 to 46% containing 0.1% TFA, and the results of amino acid analysis (Cys being not detected) and mass spectrometry of the product were consistent with the theoretical values.

[0075]

Amino Acid Analysis

Hydrolysis: 1% phenol/6N aqueous hydrochloric acid, 110°C, 8 hours;

Analysis method: the ninhydrin method;

* Reference amino acid; Theoretical values are indicated in parentheses:

	Asx: 2.77	(3)
	Val: 1.70	(2)
*	Leu: 1.00	(1)
	Tyr: 1.98	(2)
	His: 0.91	(1)

Mass spectrum (FAB)

$[M+H]^+$: 1241

[0076]

[Table 5]

Schedule 1

Steps	Duration (min) x the number of treatments
1. (washing) DMF 1.2 ml	1 x 2
2. (deprotection) 50% piperidine/DMF	12 x 1
3. (washing) DMF 1.2 ml	1 x 7
4. (coupling) each amino-protected amino acid (5 equivalents)/NMP solution 0.9 ml, DIC (5 equivalents)/NMP solution 0.3 ml	30 x 1
5. (washing) DMF 1.2 ml	1 x 2
6. (coupling) each amino-protected amino acid (5 equivalents)/NMP solution 0.9 ml, DIC (5 equivalents)/NMP solution 0.3 ml	30 x 1
7. (washing) DMF 1.2 ml	1 x 4

[0077]

(2) Synthesis of SART-3 "172-181" Leu-Phe-Glu-Lys-Ala-Val-Lys-Asp-Tyr-Ile (SEQ ID NO: 4)

According to a similar manner to that described in above (1), using 100 mg of Fmoc-Ile-Alko Resin (0.41mmol/g, 100-200mesh), Fmoc-Tyr(tBu)-OH, Fmoc-Asp(OtBu)-OH, Fmoc-Lys(Boc)-OH, Fmoc-Val-OH, Fmoc-Ala-OH, Fmoc-Lys(Boc)-OH, Fmoc-Glu(OtBu)-OH, Fmoc-Phe-OH, and Fmoc-Leu-OH were coupled in order, and the product was then deprotected. The crude peptide obtained was dissolved in aqueous acetic acid and

injected into a reverse phase packing material YMC-PACK ODS-A column (30 ϕ x 250 mm) that has been pre-equilibrated with 0.1% aqueous TFA. The column was washed with 0.1% aqueous TFA, and the elution at a flow rate of 7 ml/min was then conducted, while increasing the concentration of acetonitrile up to 30% over 300 minutes. The eluate was monitored by A 220 nm. The fractions containing the desired product were combined together and lyophilized to obtain 66.3 mg of Leu-Phe-Glu-Lys-Ala-Val-Lys-Asp-Tyr-Ile.

[0078]

The peptide obtained, Leu-Phe-Glu-Lys-Ala-Val-Lys-Asp-Tyr-Ile, had a retention time of 23.8 minutes in an analysis using a reverse phase packing material YMC-PACK ODS-AM column (4.6 ϕ x 250 mm) eluted with a linear gradient of acetonitrile concentration from 12 to 42% containing 0.1% TFA, and the results of amino acid analysis and mass spectrometry of the product were consistent with the theoretical values.

[0079]

Amino Acid Analysis

Hydrolysis: 1% phenol/6N aqueous hydrochloric acid, 110°C, 12 hours;

Analysis method: the ninhydrin method;

* Reference amino acid; Theoretical values are indicated in parentheses:

Asx: 0.94 (1)

Glx: 1.03 (1)

Ala: 1.00 (1)

Val: 0.88 (1)

Ile: 0.92 (1)

* Leu: 1.00 (1)

Tyr: 0.96 (1)

Phe: 0.97 (1)

Lys: 1.45 (2)

Mass spectrum (FAB):

$[M+H]^+$: 1225

[0080]

(3) Synthesis of SART-3 "284-292" Asn-Tyr-Asn-Lys-Ala-Leu-Gln-Gln-Leu (SEQ ID NO: 5)

According to a similar manner to that described in above (1), using 100 mg of Fmoc-Leu-Alko Resin, Fmoc-Gln-OH, Fmoc-Gln-OH, Fmoc-Leu-OH, Fmoc-Ala-OH, Fmoc-Lys(Boc)-OH, Fmoc-Asn-OH, Fmoc-Tyr(tBu)-OH, and Fmoc-Asn-OH were coupled in order, and the product was then deprotected. The crude peptide obtained was dissolved in aqueous acetic acid and injected into a reverse phase packing material YMC-PACK ODS-A column (30 ϕ x 250 mm) that has been pre-equilibrated with 0.1% aqueous TFA. The column was washed with 0.1% aqueous TFA, and the elution at a flow rate of 7 ml/min was then conducted, while increasing the concentration of acetonitrile up to 30% over 300 minutes. The eluate was monitored by A 220 nm. The fractions containing the desired product were combined together and lyophilized to obtain 25.0 mg of Asn-Tyr-Asn-Lys-Ala-Leu-Gln-Gln-Leu.

[0081]

The peptide obtained, Asn-Tyr-Asn-Lys-Ala-Leu-

Gln-Gln-Leu, had a retention time of 19.0 minutes in an analysis using a reverse phase packing material YMC-PACK ODS-AM column (4.6 ϕ x 250 mm) eluted with a linear gradient of acetonitrile concentration from 12 to 42% containing 0.1% TFA, and the results of amino acid analysis and mass spectrometry of the product were consistent with the theoretical values.

[0082]

Amino Acid Analysis

Hydrolysis: 1% phenol/6N aqueous hydrochloric acid, 110°C, 12 hours;

Analysis method: the ninhydrin method;

* Reference amino acid; Theoretical values are indicated in parentheses:

Asx: 1.87 (2)

Glx: 2.03 (2)

Ala: 0.98 (1)

* Leu: 2.00 (2)

Tyr: 0.99 (1)

Lys: 0.97 (1)

Mass spectrum (FAB):

$[M+H]^+$: 1091

[0083]

<u>(4) Synthesis of SART-3 "315-323" Ala-Tyr-Ile-Asp-Phe-Glu-Met-Lys-Ile (SEQ ID NO: 6)</u>

According to a similar manner to that described in above (1), using 100 mg of Fmoc-Ile-Alko Resin (0.62mmol/g, 100-200mesh), Fmoc-Lys(Boc)-OH, Fmoc-Met-OH, Fmoc-

Glu(OtBu)-OH, Fmoc-Phe-OH, Fmoc-Asp(OtBu)-OH, Fmoc-Ile-OH, Fmoc-Tyr(tBu)-OH, and Fmoc-Ala-OH were coupled in order, and the product was then deprotected. The crude peptide obtained was dissolved in aqueous acetic acid and injected into a reverse phase packing material YMC-PACK ODS-A column (30 ϕ x 250 mm) that has been pre-equilibrated with 0.1% aqueous TFA. The column was washed with 0.1% aqueous TFA, and the elution at a flow rate of 7 ml/min was then conducted, while increasing the concentration of acetonitrile up to 40% over 180 minutes. The eluate was monitored by A 220 nm. The fractions containing the desired product were combined together and lyophilized to obtain 15.4 mg of Ala-Tyr-Ile-Asp-Phe-Glu-Met-Lys-Ile.

[0084]

The peptide obtained, Ala-Tyr-Ile-Asp-Phe-Glu-Met-Lys-Ile, had a retention time of 19.6 minutes in an analysis using a reverse phase packing material YMC-PACK ODS-AM column (4.6 ϕ x 250 mm) eluted with a linear gradient of acetonitrile concentration from 21 to 51% containing 0.1% TFA, and the results of amino acid analysis (Met being not detected) and mass spectrometry of the product were consistent with the theoretical values.

[0085]

Amino Acid Analysis

Hydrolysis: 1% phenol/6N aqueous hydrochloric acid, 110°C, 12 hours;

Analysis method: the ninhydrin method;

* Reference amino acid; Theoretical values are indicated in

parentheses:

Asx: 0.91 (1)

Glx: 1.06 (1)

Ala: 1.06 (1)

Ile: 1.69 (2)

Tyr: 0.81 (1)

* Phe: 1.00 (1)

Lys: 0.87 (1)

Mass spectrum (FAB):

$[M+H]^+$: 1130

[0086]

(5) Synthesis of SART-3 "416-425" Asp-Tyr-Val-Glu-Ile-Trp-Gln-Ala-Tyr-Leu (SEQ ID NO: 7)

According to a similar manner to that described in above (1), using 100 mg of Fmoc-Leu-Alko Resin, Fmoc-Tyr(tBu)-OH, Fmoc-Ala-OH, Fmoc-Gln-OH, Fmoc-Trp(Boc)-OH, Fmoc-Ile-OH, Fmoc-Glu(OtBu)-OH, Fmoc-Val-OH, Fmoc-Tyr(tBu)-OH, and Fmoc-Asp(OtBu)-OH were coupled in order, and the product was then deprotected. The crude peptide obtained was dissolved in aqueous acetic acid and injected into a reverse phase packing material YMC-PACK ODS-A column (30 ϕ x 250 mm) that has been pre-equilibrated with 0.1% aqueous TFA. The column was washed with 0.1% aqueous TFA, and the elution at a flow rate of 7 ml/min was then conducted, while increasing the concentration of acetonitrile up to 35% over 180 minutes. The eluate was monitored by A 220 nm. The fractions containing the desired product were combined together and lyophilized to obtain 18.9

mg of Asp-Tyr-Val-Glu-Ile-Trp-Gln-Ala-Tyr-Leu.

[0087]

The peptide obtained, Asp-Tyr-Val-Glu-Ile-Trp-Gln-Ala-Tyr-Leu, had a retention time of 20.5 minutes in an analysis using a reverse phase packing material YMC-PACK ODS-AM column (4.6 ϕ x 250 mm) eluted with a linear gradient of acetonitrile concentration from 25 to 55% containing 0.1% TFA, and the results of amino acid analysis (Trp being not detected) and mass spectrometry of the product were consistent with the theoretical values.

[0088]

Amino Acid Analysis

Hydrolysis: 1% phenol/6N aqueous hydrochloric acid, 110°C, 10 hours;

Analysis method: the ninhydrin method;

* Reference amino acid; Theoretical values are indicated in parentheses:

Asx: 1.00 (1)

Glx: 2.09 (2)

Ala: 1.04 (1)

Val: 0.89 (1)

Ile: 0.86 (1)

* Leu: 1.00 (1)

Tyr: 1.95 (2)

Mass spectrum (FAB):

$[M+H]^+$: 1300

[0089]

(6) Synthesis of SART-3 "426-434" Asp-Tyr-Leu-Arg-Arg-Arg-Val-Asp-Phe (SEQ ID NO: 8)

According to a similar manner to that described in above (1), using 100 mg of Fmoc-Phe-Alko Resin (0.72mmol/g, 100-200mesh), Fmoc-Asp(OtBu)-OH, Fmoc-Val-OH, Fmoc-Arg(Pmc)-OH, Fmoc-Arg(Pmc)-OH, Fmoc-Arg(Pmc)-OH, Fmoc-Leu-OH, Fmoc-Tyr(tBu)-OH, and Fmoc-Asp(OtBu)-OH were coupled in order, and the product was then deprotected. The crude peptide obtained was dissolved in aqueous acetic acid and injected into a reverse phase packing material YMC-PACK ODS-A column (30 ϕ x 250 mm) that has been pre-equilibrated with 0.1% aqueous TFA. The column was washed with 0.1% aqueous TFA, and the elution at a flow rate of 7 ml/min was then conducted, while increasing the concentration of acetonitrile up to 25% over 240 minutes. The eluate was monitored by A 220 nm. The fractions containing the desired product were combined together and lyophilized to obtain 34.0 mg of Asp-Tyr-Leu-Arg-Arg-Arg-Val-Asp-Phe.

[0090]

The peptide obtained, Asp-Tyr-Leu-Arg-Arg-Arg-Val-Asp-Phe, had a retention time of 20.1 minutes in an analysis using a reverse phase packing material YMC-PACK ODS-AM column (4.6 ϕ x 250 mm) eluted with a linear gradient of acetonitrile concentration from 12 to 42% containing 0.1% TFA, and the results of amino acid analysis and mass spectrometry of the product were consistent with the theoretical values.

[0091]

Amino Acid Analysis

Hydrolysis: 1% phenol/6N aqueous hydrochloric acid, 110°C, 12 hours;

Analysis method: the ninhydrin method;

* Reference amino acid; Theoretical values are indicated in parentheses:

Asx: 1.90 (2)

Val: 0.95 (1)

* Leu: 1.00 (1)

Tyr: 1.00 (1)

Phe: 0.99 (1)

Arg: 2.93 (3)

Mass spectrum (FAB):

$[M+H]^+$: 1239

[0092]

(7) Synthesis of SART-3 "448-456" Ala-Phe-Thr-Arg-Ala-Leu-Glu-Tyr-Leu (SEQ ID NO: 9)

According to a similar manner to that described in above (1), using 100 mg of Fmoc-Leu-Alko Resin, Fmoc-Tyr(tBu)-OH, Fmoc-Glu(OtBu)-OH, Fmoc-Leu-OH, Fmoc-Ala-OH, Fmoc-Arg(Pmc)-OH, Fmoc-Thr(tBu)-OH, Fmoc-Phe-OH, and Fmoc-Ala-OH were coupled in order, and the product was then deprotected. The crude peptide obtained was dissolved in aqueous acetic acid and injected into a reverse phase packing material YMC-PACK ODS-A column (30 ϕ x 250 mm) that has been pre-equilibrated with 0.1% aqueous TFA. The column was washed with 0.1% aqueous TFA, and the elution at a flow rate of 7 ml/min was then conducted, while increasing the concentration of

acetonitrile up to 30% over 240 minutes. The eluate was monitored by A 220 nm. The fractions containing the desired product were combined together and lyophilized to obtain 22.8 mg of Ala-Phe-Thr-Arg-Ala-Leu-Glu-Tyr-Leu.

[0093]

The peptide obtained, Ala-Phe-Thr-Arg-Ala-Leu-Glu-Tyr-Leu, had a retention time of 18.1 minutes in an analysis using a reverse phase packing material YMC-PACK ODS-AM column (4.6 ϕ x 250 mm) eluted with a linear gradient of acetonitrile concentration from 20 to 50% containing 0.1% TFA, and the results of amino acid analysis and mass spectrometry of the product were consistent with the theoretical values.

[0094]

Amino Acid Analysis

Hydrolysis: 1% phenol/6N aqueous hydrochloric acid, 110°C, 12 hours;

Analysis method: the ninhydrin method;

* Reference amino acid; Theoretical values are indicated in parentheses:

Thr: 0.91 (1)

Glx: 1.03 (1)

Ala: 1.91 (2)

* Leu: 2.00 (2)

Tyr: 1.00 (1)

Phe: 0.97 (1)

Arg: 0.97 (1)

Mass spectrum (FAB):

$[M+H]^+$: 1083

[0095]

Example 6

CTL induction from Peripheral Blood Lymphocytes by Tumor Antigen Peptides and Derivatives thereof

The peptides "109-118" (SEQ ID NO: 3) and "315-323" (SEQ ID NO: 6) synthesized as shown in Example 5 were investigated for their ability to induce antigen-specific CTLs from peripheral blood lymphocytes.

Using the Ficoll method, lymphocytes were separated from peripheral blood of healthy donors who were heterozygous for A24 in the HLA-A locus (referred to as HD1 and HD2, respectively). The lymphocytes were placed into wells of a 24-well plate at $2x10^6$ cells/well, and cultured in the lymphocyte medium. The above tumor antigen peptides were added to the culture medium at 10 μM to stimulate the peripheral blood lymphocytes. After one week, the above tumor antigen peptide was added to attain 10 μM together with about $2x10^5$ cells of X-radiated (50 Gy) peripheral blood lymphocytes for the second stimulation. After additional one week, the third stimulation was conducted in a similar manner. Cultured lymphocytes were harvested one week after the third stimulation. Using as target cells ($1x10^4$ cells) MT-2, which is an HLA-A2402-positive leukemia T cell line expressing SART-3, and RPMI8402, which is an HLA-A2402-negative leukemia T cell line expressing SART-3, the amount of IFN-γ in the culture medium produced by the above lymphocytes ($8x10^4$ cells) in response to the target cells

was measured in accordance with a similar ELISA method to that in Example 1. The results are shown in Table 6.

[0096]

[Table 6]

	IFN-γ in Supernatant (pg/ml)			
	HD1		HD2	
Antigen Peptides	MT-2	RPMI8402	MT-2	RPMI8402
"109-118"	1771	159	2078	28
"315-323"	2041	26	974	40
None	552	154	413	69

[0097]

Peripheral blood lymphocytes stimulated with "109-118" and "315-323" peptides reacted to MT-2 (HLA-A24-positive) but not to RPMI8402 (HLA-A24-negative), indicating that CTLs specific for tumor antigen peptide were induced in a HLA-A24-restricted manner.

Likewise, a similar experiment can be conducted using COS-7 cells (ATCC No. CRL1651) or VA-13 cells (RIKEN CELL BANK, The Institute of Physical and Chemical Research) into which an expression plasmid for HLA-A24 cDNA has been introduced and which have been pulsed with the above peptides, instead of MT-2 used in the present experiment (*J. Exp. Med.*, 187:277, 1998).

[0098]

SEQUENCE LISTING FREE TEXT

In the amino acid sequence shown in SEQ ID NO: 25, the second amino acid is phenylalanine, tyrosine, methionine,

or tryptophan, and the tenth amino acid is phenylalanine, leucine, isoleucine, tryptophan, or methionine.

[0099]

In the amino acid sequence shown in SEQ ID NO: 26, the second amino acid is phenylalanine, tyrosine, methionine, or tryptophan, and the tenth amino acid is phenylalanine, leucine, isoleucine, tryptophan, or methionine.

[0100]

In the amino acid sequence shown in SEQ ID NO: 27, the second amino acid is phenylalanine, tyrosine, methionine, or tryptophan, and the ninth amino acid is phenylalanine, leucine, isoleucine, tryptophan, or methionine.

[0101]

In the amino acid sequence shown in SEQ ID NO: 28, the second amino acid is phenylalanine, tyrosine, methionine, or tryptophan, and the ninth amino acid is phenylalanine, leucine, isoleucine, tryptophan, or methionine.

[0102]

In the amino acid sequence shown in SEQ ID NO: 29, the second amino acid is phenylalanine, tyrosine, methionine, or tryptophan, and the tenth amino acid is phenylalanine, leucine, isoleucine, tryptophan, or methionine.

[0103]

In the amino acid sequence shown in SEQ ID NO: 30, the second amino acid is phenylalanine, tyrosine, methionine, or tryptophan, and the ninth amino acid is phenylalanine, leucine, isoleucine, tryptophan, or methionine.

[0104]

In the amino acid sequence shown in SEQ ID NO: 31, the second amino acid is phenylalanine, tyrosine, methionine, or tryptophan, and the ninth amino acid is phenylalanine, leucine, isoleucine, tryptophan, or methionine.

[0105]

Effect of the invention:

According to the present invention, a novel tumor antigen protein and gene therefor, tumor antigen peptides derived from said tumor antigen protein, and derivatives thereof, as well as medicaments, or prophylactics for tumors using such tumor antigen protein, gene, tumor antigen peptides, or derivatives thereof *in vivo* or *in vitro*, can be provided.

[0106]

SEQUENCE LISTING

<110> ITOH, Kyogo; Sumitomo Pharmaceuticals Company, Limited

<120> A Novel Tumor Antigen SART-3, and It's Tumor Antigen Peptides

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[0107]

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Lys Thr Met Gly Pro Ala Trp Asp Gln Gln Glu Glu Gly Val Ser Glu
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Ser Asp Gly Asp Glu Tyr Ala Met Ala Ser Ser Ala Glu Ser Ser Pro
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Gly Glu Tyr Glu Trp Glu Tyr Asp Glu Glu Glu Glu Lys Asn Gln Leu
85 90 95

Glu Ile Glu Arg Leu Glu Glu Gln Leu Ser Ile Asn Val Tyr Asp Tyr
100 105 110

Asn Cys His Val Asp Leu Ile Arg Leu Leu Arg Leu Glu Gly Glu Leu

115 120 125

Thr Lys Val Arg Met Ala Arg Gln Lys Met Ser Glu Ile Phe Pro Leu

130 135 140

Thr Glu Glu Leu Trp Leu Glu Trp Leu His Asp Glu Ile Ser Met Ala

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Gln Asp Gly Leu Asp Arg Glu His Val Tyr Asp Leu Phe Glu Lys Ala

165 170 175

Val Lys Asp Tyr Ile Cys Pro Asn Ile Trp Leu Glu Tyr Gly Gln Tyr

180 185 190

Ser Val Gly Gly Ile Gly Gln Lys Gly Gly Leu Glu Lys Val Arg Ser

195 200 205

Val Phe Glu Arg Ala Leu Ser Ser Val Gly Leu His Met Thr Lys Gly

210 215 220

Leu Ala Leu Trp Glu Ala Tyr Arg Glu Phe Glu Ser Ala Ile Val Glu

225 230 235 240

Ala Ala Arg Leu Glu Lys Val His Ser Leu Phe Arg Arg Gln Leu Ala

245 250 255

Ile Pro Leu Tyr Asp Met Glu Ala Thr Phe Ala Glu Tyr Glu Glu Trp

260 265 270

Ser Glu Asp Pro Ile Pro Glu Ser Val Ile Gln Asn Tyr Asn Lys Ala

275 280 285

Leu Gln Gln Leu Glu Lys Tyr Lys Pro Tyr Glu Glu Ala Leu Leu Gln

290 295 300

Ala Glu Ala Pro Arg Leu Ala Glu Tyr Gln Ala Tyr Ile Asp Phe Glu

305 310 315 320

Met Lys Ile Gly Asp Pro Ala Arg Ile Gln Leu Ile Phe Glu Arg Ala

325 330 335

Leu Val Glu Asn Cys Leu Val Pro Asp Leu Trp Ile Arg Tyr Ser Gln
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Asn Arg Ala Ile Arg Asn Cys Pro Trp Thr Val Ala Leu Trp Ser Arg
370 375 380
Tyr Leu Leu Ala Met Glu Arg His Gly Val Asp His Gln Val Ile Ser
385 390 395 400
Val Thr Phe Glu Lys Ala Leu Asn Ala Gly Phe Ile Gln Ala Thr Asp
405 410 415
Tyr Val Glu Ile Trp Gln Ala Tyr Leu Asp Tyr Leu Arg Arg Arg Val
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Asp Phe Lys Gln Asp Ser Ser Lys Glu Leu Glu Glu Leu Arg Ala Ala
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Phe Thr Arg Ala Leu Glu Tyr Leu Lys Gln Glu Val Glu Glu Arg Phe
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465 470 475 480
Ile Glu Ala Arg Leu Cys Asn Asn Met Gln Lys Ala Arg Glu Leu Trp
485 490 495
Asp Ser Ile Met Thr Arg Gly Asn Ala Lys Tyr Ala Asn Met Trp Leu
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Glu Tyr Tyr Asn Leu Glu Arg Ala His Gly Asp Thr Gln His Cys Arg
515 520 525
Lys Ala Leu His Arg Ala Val Gln Cys Thr Ser Asp Tyr Pro Glu His
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Val Cys Glu Val Leu Leu Thr Met Glu Arg Thr Glu Gly Ser Leu Glu

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Asp Trp Asp Ile Ala Val Gln Lys Thr Glu Thr Arg Leu Ala Arg Val

565 570 575

Asn Glu Gln Arg Met Lys Ala Ala Glu Lys Glu Ala Ala Leu Val Gln

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Gln Glu Glu Glu Lys Ala Glu Gln Arg Lys Arg Ala Arg Ala Glu Lys

595 600 605

Lys Ala Leu Lys Lys Lys Lys Lys Ile Arg Gly Pro Glu Lys Arg Gly

610 615 620

Ala Asp Glu Asp Asp Glu Lys Glu Trp Gly Asp Asp Glu Glu Glu Gln

625 630 635 640

Pro Ser Lys Arg Arg Arg Val Glu Asn Ser Ile Pro Ala Ala Gly Glu

645 650 655

Thr Gln Asn Val Glu Val Ala Ala Gly Pro Ala Gly Lys Cys Ala Ala

660 665 670

Val Asp Val Glu Pro Pro Ser Lys Gln Lys Glu Lys Ala Ala Ser Leu

675 680 685

Lys Arg Asp Met Pro Lys Val Leu His Asp Ser Ser Lys Asp Ser Ile

690 695 700

Thr Val Phe Val Ser Asn Leu Pro Tyr Ser Met Gln Glu Pro Asp Thr

705 710 715 720

Lys Leu Arg Pro Leu Phe Glu Ala Cys Gly Glu Val Val Gln Ile Arg

725 730 735

Pro Ile Phe Ser Asn Arg Gly Asp Phe Arg Gly Tyr Cys Tyr Val Glu

740 745 750

Phe Lys Glu Glu Lys Ser Ala Leu Gln Ala Leu Glu Met Asp Arg Lys

755 760 765

Ser Val Glu Gly Arg Pro Met Phe Val Ser Pro Cys Val Asp Lys Ser
770 775 780
Lys Asn Pro Asp Phe Lys Val Phe Arg Tyr Ser Thr Ser Leu Glu Lys
785 790 795 800
His Lys Leu Phe Ile Ser Gly Leu Pro Phe Ser Cys Thr Lys Glu Glu
805 810 815
Leu Glu Glu Ile Cys Lys Ala His Gly Thr Val Lys Asp Leu Arg Leu
820 825 830
Val Thr Asn Arg Ala Gly Lys Pro Lys Gly Leu Ala Tyr Val Glu Tyr
835 840 845
Glu Asn Glu Ser Gln Ala Ser Gln Ala Val Met Lys Met Asp Gly Met
850 855 860
Thr Ile Lys Glu Asn Ile Ile Lys Val Ala Ile Ser Asn Pro Pro Gln
865 870 875 880
Arg Lys Val Pro Glu Lys Pro Glu Thr Arg Lys Ala Pro Gly Gly Pro
885 890 895
Met Leu Leu Pro Gln Thr Tyr Gly Ala Arg Gly Lys Gly Arg Thr Gln
900 905 910
Leu Ser Leu Leu Pro Arg Ala Leu Gln Arg Pro Ser Ala Ala Ala Pro
915 920 925
Gln Ala Glu Asn Gly Pro Ala Ala Ala Pro Ala Val Ala Ala Pro Ala
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[0108]

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gct gag tcc aag gct ggg ccc aag gct gac gga gag gag gat gag gtt 98
Ala Glu Ser Lys Ala Gly Pro Lys Ala Asp Gly Glu Glu Asp Glu Val
15 20 25

aag gcg gct agg aca agg aga aag gtg tta tcg cgg gct gtg gcc gct 146
Lys Ala Ala Arg Thr Arg Arg Lys Val Leu Ser Arg Ala Val Ala Ala
30 35 40 45

gcg aca tac aag acc atg ggg cca gcg tgg gat cag cag gag gaa ggc 194
Ala Thr Tyr Lys Thr Met Gly Pro Ala Trp Asp Gln Gln Glu Glu Gly
50 55 60

gtg agc gag agc gat ggg gat gag tac gcc atg gct tcc tcc gcg gag 242
Val Ser Glu Ser Asp Gly Asp Glu Tyr Ala Met Ala Ser Ser Ala Glu
65 70 75

agc tcc ccc ggg gag tac gag tgg gaa tat gac gaa gag gag gag aaa 290
Ser Ser Pro Gly Glu Tyr Glu Trp Glu Tyr Asp Glu Glu Glu Glu Lys
80 85 90

aac cag ctg gag att gag aga ctg gag gag cag ttg tct atc aac gtc 338
Asn Gln Leu Glu Ile Glu Arg Leu Glu Glu Gln Leu Ser Ile Asn Val
95 100 105

tat gac tac aac tgc cat gtg gac ttg atc aga ctg ctc agg ctg gaa 386

Tyr Asp Tyr Asn Cys His Val Asp Leu Ile Arg Leu Leu Arg Leu Glu
110 115 120 125

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Gly Glu Leu Thr Lys Val Arg Met Ala Arg Gln Lys Met Ser Glu Ile
130 135 140

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Phe Pro Leu Thr Glu Glu Leu Trp Leu Glu Trp Leu His Asp Glu Ile
145 150 155

agc atg gcc cag gat ggc ctg gac aga gag cac gtg tat gac ctc ttt 530
Ser Met Ala Gln Asp Gly Leu Asp Arg Glu His Val Tyr Asp Leu Phe
160 165 170

gag aaa gcc gtg aag gat tac att tgt cct aac att tgg cta gag tat 578
Glu Lys Ala Val Lys Asp Tyr Ile Cys Pro Asn Ile Trp Leu Glu Tyr
175 180 185

ggc cag tac tca gtt ggt ggg att ggt cag aaa ggt ggc ctt gag aaa 626
Gly Gln Tyr Ser Val Gly Gly Ile Gly Gln Lys Gly Gly Leu Glu Lys
190 195 200 205

gtt cgc tcc gtg ttt gaa agg gct ctc tcg tct gtt ggt tta cat atg 674
Val Arg Ser Val Phe Glu Arg Ala Leu Ser Ser Val Gly Leu His Met
210 215 220

acc aaa gga ctc gcc ctc tgg gag gct tac cga gag ttt gaa agt gcg 722
Thr Lys Gly Leu Ala Leu Trp Glu Ala Tyr Arg Glu Phe Glu Ser Ala
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Tyr Ser Gln Tyr Leu Asp Arg Gln Leu Lys Val Lys Asp Leu Val Leu
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Ser Val His Asn Arg Ala Ile Arg Asn Cys Pro Trp Thr Val Ala Leu
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Trp Ser Arg Tyr Leu Leu Ala Met Glu Arg His Gly Val Asp His Gln
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Document name: Abstract

Summary:

Object: To provide a novel tumor antigen protein and gene therefor, tumor antigen peptides derived from said tumor antigen protein or derivatives thereof as well as medicaments, or prophylactics for tumors using such tumor substances *in vitro* or in vitro.

Solution: A novel tumor antigen protein and gene therefor, tumor antigen peptides derived from said tumor antigen protein, and derivatives thereof, as well as medicaments, or prophylactics for tumors using such tumor antigen protein, gene, tumor antigen peptides, or derivatives thereof *in vivo* or *in vitro*.

Selected figure: None